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Conformational Behaviour of Azasugars Based on Mannuronic Acid

Erwin R. van Rijssel,^[a] Antonius P. A. Janssen,^[a] Alexandra Males,^[b] Gideon J. Davies,^[b] Gijsbert A. van der Marel,^[a] Herman S. Overkleef,^{*,[a]} and Jeroen D. C. Codée^{*,[a]}

Dedicated to the memory of Professor Dr. Werner Reutter.

A set of mannuronic-acid-based iminosugars, consisting of the C-5-carboxylic acid, methyl ester and amide analogues of 1-deoxymannorjirimycin (DMJ), was synthesised and their pH-dependent conformational behaviour was studied. Under acidic conditions the methyl ester and the carboxylic acid adopted an “inverted” ¹C₄ chair conformation as opposed to the “normal” ⁴C₁ chair at basic pH. This conformational change is explained in terms of the stereoelectronic effects of the ring substituents and it parallels the behaviour of the mannuronic

acid ester oxocarbenium ion. Because of this solution-phase behaviour, the mannuronic acid ester azasugar was examined as an inhibitor for a *Caulobacter* GH47 mannosidase that hydrolyses its substrates by way of a reaction itinerary that proceeds through a ³H₄ transition state. No binding was observed for the mannuronic acid ester azasugar, but sub-atomic resolution data were obtained for the DMJ-CkGH47 complex, showing two conformations—³S₁ and ¹C₄—for the DMJ inhibitor.

Introduction

Stereoelectronic substituent effects have a profound effect on the three-dimensional structures of molecules. Whereas substituents on a cyclic compound generally have a preference for (pseudo)equatorial positions for steric reasons, the electronic spatial preferences depend on different forces such as charge–charge and dipole–dipole interactions.^[1] The conformation and reactivity of carbohydrates are determined to a large extent by the natures and orientations of the substituents. This influence becomes apparent in glycosylation reactions, in which the amount, nature and orientation of the hydroxy groups, protected with electron-withdrawing esters or more electron-neutral ether groups, determine the overall reactivity.^[2]

It has long been known that, in glycosylations, axial substituents are less deactivating or “disarming” than their equatorially

positioned equivalents.^[3] Similarly, the basicity of iminosugars (or “azasugars”), carbohydrates in which the endocyclic oxygen atom is replaced by an amine group, is influenced by the orientation of the ring substituents, with azasugars bearing more axially positioned hydroxy groups being more basic than their stereoisomers bearing equatorially positioned substituents.^[4] These effects can be explained in terms of more favourable interaction of the axially positioned electronegative oxygen substituents with the positive charge present on the azasugar ring in a protonated state and the (partial) positive charge of oxocarbenium ion (-like) intermediates in glycosylation reactions.^[4–7]

In mannuronic acids, mannositides in which the C6-OH group is oxidised to a carboxylic acid functionality, the carboxylic acid has a profound effect on the conformation and reactivity of the pyranoside.^[8] In the context of the construction of bacterial oligosaccharides we have studied the glycosylation behaviour of a variety of mannuronic acid donors in detail and we have found these to be unexpectedly reactive.^[9] In addition, glycosylations involving these donors proceed with an extraordinary selectivity to provide 1,2-*cis* glycosidic linkages. These findings were explained in terms of the conformational preferences of (partially) positively charged mannuronic acid oxocarbenium ion (-like) intermediates that are governed by the ring substituent effects. These species prefer to adopt a “flipped” ring structure and in the ³H₄ (-like) oxocarbenium ion all substituents take up the most stabilising (or least destabilising) orientation: the C2-OR pseudoequatorial and the C3-, C4-OR and C5-COOR groups pseudoaxial. Indeed, DFT calculations indicate that the ³H₄ oxocarbenium ion is significantly more stable than the alternative (“non-ring-flipped”) ⁴H₃ ion (Scheme 1A).^[10]

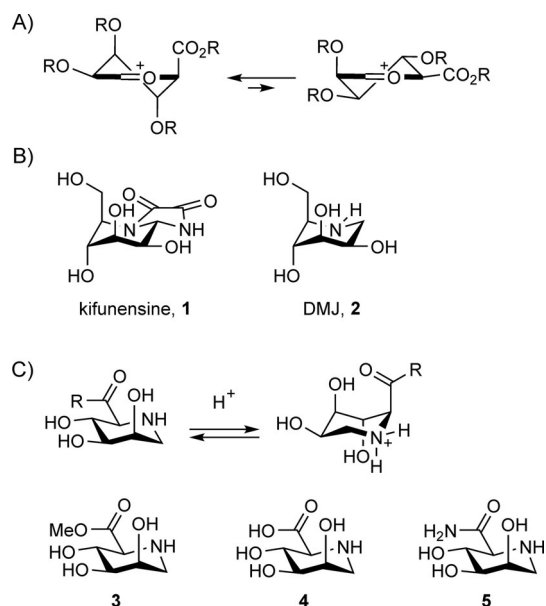
[a] Dr. E. R. van Rijssel, A. P. A. Janssen, Prof. Dr. G. A. van der Marel, Prof. Dr. H. S. Overkleef, Dr. J. D. C. Codée
Leiden Institute of Chemistry, Leiden University
Einsteinweg 55, 2333 CC Leiden (The Netherlands)
E-mail: jcodee@chem.leidenuniv.nl
h.s.overkleef@chem.leidenuniv.nl

[b] A. Males, Prof. Dr. G. J. Davies
York Structural Biology Laboratory
Department of Chemistry, The University of York
York YO10 5DD (UK)

Supporting information and the ORCID identification numbers for the authors of this article can be found under <http://dx.doi.org/10.1002/cbic.201700080>.

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Scheme 1. A) Conformational equilibrium for the mannuronic acid oxocarbenium ion. B) Structures of the mannosidase inhibitors kifunensine (1) and 1-deoxymannojirimycin (DMJ, 2) in the 1C_4 conformation. C) Mannuronic acid azasugars studied here.

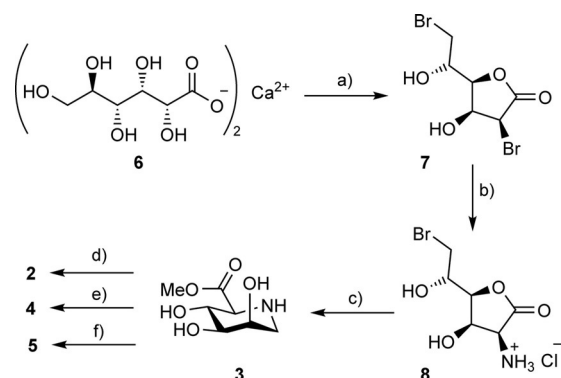
Carbohydrate-processing enzymes, such as glycoside hydrolases, may induce a chemical transformation by forcing the carbohydrate substrate into an unusual conformation.^[11] α -Mannosidases that belong to the CAZY family GH47 are inverting glycoside hydrolases that cleave α -1,2-mannosidic linkages. The mammalian GH47 mannosidases can be found in the Golgi and endoplasmic reticulum (ER), where they cleave mannose residues from N-glycans, thereby playing an important role in protein biosynthesis and quality control. The mechanism by which these hydrolases cleave the 1,2-mannosidic bonds is notable because they employ an unusual catalytic itinerary. The substrate that is to be cleaved binds in a ${}^{3,0}B/{}^2S_1$ conformation and is hydrolysed in a reaction that proceeds through a transition state in which the mannose ring adopts a 3H_4 conformation.^[12] Kifunesine (1, Scheme 1B), a potent inhibitor of the mannosidase I enzyme, has been shown to adopt a ring-flipped 1C_4 conformation, and a similar conformation was found for 1-deoxymannojirimycin (DMJ, 2, Scheme 1B) bound in the active site of *Saccharomyces cerevisiae*.^[12]

We were inspired by the conformations of the inhibitors of the GH47 enzymes to explore the behaviour of azasugars based on mannuronic acid. Here we report on the synthesis of mannuronic-acid-based azasugars 3, 4 and 5 (Scheme 1C) and their conformational behaviour. We show that the stereoelectronic effects that determine the structures of the mannuronic acid oxocarbenium ions also impact the three-dimensional structures of these azasugars and that protonation of the ring nitrogen can induce a ring flip leading to an axial-rich 1C_4 conformation in solution. We build on this to show how deoxymannojirimycin, the “parent” compound, binds to a bacterial GH47 enzyme from *Caulobacter* sp. K31^[12] but also that—un-

fortunately, despite improved solution behaviour—the mannuronic acid derivatives do not bind to the GH47 enzyme, likely by virtue of their altered C5-substituents.

Results and Discussion

The synthesis of DMJ (2) and its C5 analogues was achieved according to the route devised by Wrodnigg and co-workers.^[13] As depicted in Scheme 2, methyl mannuronic acid ester azasugar 3 was obtained in four steps from the commercially available calcium D-gluconate monohydrate (6).^[14] The gluconate 6



Scheme 2. Synthesis of DMJ and its C5 analogues for this study. Reaction conditions: a) i: HBr, AcOH; ii: MeOH, 26% over two steps; b) i: NaN₃, acetone; ii: H₂, Pd/C, HCl (aq.), MeOH, 55% over two steps; c) i: Et₃N, MeOH, quant.; ii: HMDS, CuSO₄·H₂O (cat.), CAN; iii: MeOH, AcCl (cat), 70% over three steps; d) NaBH₄, EtOH, 29%; e) NaOH, H₂O, quant.; f) NH₃, MeOH, quant.

was treated with HBr in acetic acid to form 3,5-di-O-acetyl-2,6-dibromo-2,6-dideoxy-D-manno-1,4-lactone after a series of acid-catalysed transformations (i.e., substitution of the C2 and C6 hydroxy groups, intramolecular ring closure and acetylation of the remaining hydroxy groups). Next, the acetyl groups at O3 and O5 were removed in an acid-catalysed transesterification with methanol to provide the pure dibromolactone 7 after crystallisation from chloroform/water, in 26% yield over the two steps. Regioselective displacement of the C2 bromide with an azide occurred with retention of configuration, as explained by Bock et al.^[15] with epimerisation of the C2-bromide to the more reactive glucose-configured dibromide and subsequent regioselective substitution by the azide. Thereafter, palladium-catalysed reduction of the intermediate azide and subsequent crystallisation from ethanol gave 2-amino-6-bromolactone 8 as its hydrochloric acid salt in 55% yield. Treatment of this salt with triethylamine in methanol led to ring opening and intramolecular bromide displacement by the C2 amine to give crude azasugar methyl ester 3.

Purification of this compound from the triethylammonium and sodium salts formed in the reaction proved difficult, because of the high polarity of the compound as well as the lability of the methyl ester towards hydrolysis. Attempts to crystallise the compound were to no avail. Therefore, all of the hydroxy groups in 3 were capped with trimethylsilyl groups^[16] to allow for the purification of the compound by chromatogra-

phy. After desilylation, the pure methyl ester **3** was obtained as its hydrochloric acid salt.

DMJ (**2**) was synthesised from **3** by a sodium-borohydride-mediated reduction and was obtained in 29% yield after column chromatography. D-Mannuronic acid azasugar **4** and amide **5** were obtained from **3** through saponification with sodium hydroxide or aminolysis with methanolic ammonia, respectively.

With the set of azasugars to hand we established their pK_a values by titration and investigated their conformational behaviour at different pH^* (the pH measured in D_2O) values by NMR spectroscopy. Table 1 summarises the results of these studies. For DMJ a pK_a value of 7.4 was measured, in line with the pK_a previously established for this compound (7.5).^[4c] The pK_a values of methyl ester **3**, amino acid **4** and amide **5** were determined to be 5.3, 7.5 and 5.8, respectively. The drop in pK_a value for the ester and the amide is a clear manifestation of the electron-withdrawing effect of the carboxylic acid ester and amide functionalities. At higher pH^* , at which acid **4** is deprotonated, the electron-withdrawing effect of the carboxylate is lowered because of its negative charge.

Table 1. pK_a values for compounds **2–5** and observed and calculated coupling constants and determined 4C_1 : 1C_4 conformer ratio.

Compound	pH*	J_{3-4} (obs.)	J_{3-4} (calcd)	NMR ratio	pK_a	
			4C_1 1C_4	4C_1 : 1C_4		
2	9	9.5	9.0	3.3	100:0	7.4
	2	9.5	9.4	4.3	100:0	
3	8	9.4	9.4	3.9	99:1	5.3
	2	7.5	9.5	4.9	56:44	
4	11	9.8	9.3	3.1	100:0	7.5
	5	8.8	9.1	3.8	94:6	
	1	8.3	9.5	4.7	75:25	
5	9	9.7	9.0	2.8	100:0	5.8
	2	9.3	9.1	4.7	100:0	
3	CD ₃ OD	9.2	9.3	4.2	99:1	
	CD ₃ OD (+ TFA)	4.8	9.3	4.7	1:99	

Figures 1–4 show the 1H NMR spectra of azasugars **2–5** recorded at varying pH^* values. In Figure 1, 1H NMR spectra of DMJ (**2**) in D_2O at pH^* 1–12 are collected. From pH^* 1 to pH^* 6.5 no changes are observed either in chemical shifts or in coupling constants. The coupling constants are indicative of a “normal” 4C_1 chair conformation for the azasugar ring. On going from pH^* 6.5 to pH^* 12 significant shifts in chemical shift are observed for all ring protons, with the direct neighbours of the amino group experiencing the largest shifts. No changes in the coupling constants of the ring protons are observed, thus indicating that no major conformation change takes place.

In Figure 2, the 1H NMR spectra of methyl ester **3** at different pH^* values are displayed. Because hydrolysis of the methyl ester was observed above pH^* 8, no spectra were recorded above this pH^* . Large chemical shift changes are seen with increasing pH^* . Especially, H5 shows a large chemical shift change and shifts from $\delta=4.04$ at pH^* 2 to 3.22 at pH^* 8. In addition, a change in coupling constants is observed for the ring protons. For example, $J_{3,4}$ changes from 9.4 Hz at basic pH^* to 7.5 Hz at acidic pH^* , indicative of a change in conformation of the azasugar ring. At high pH^* the azasugar adopts a single conformation, whereas both the 1C_4 and 4C_1 conformers are present at low pH^* (vide infra).

Mannuronic acid **4** can exist in three different charged states: the fully protonated state, the neutral zwitterionic state and the negatively charged state. Figure 3 shows 1H NMR spectra of **4** from pH^* 1 to pH^* 12. Again, large chemical shift changes are observed with changing pH^* (especially for H5, which shows a shift from $\delta=3.9$ to $\delta=2.9$ ppm). A small change in coupling constants is also apparent: $J_{3,4}$ changes from 9.8 Hz at high pH^* to 8.8 Hz at neutral pH^* to 8.3 Hz at acidic pH^* . Thus, in line with the conformational behaviour of methyl ester **3**, mannuronic acid **4** can change its conformation in a pH-dependent manner.

Figure 4 displays a set of 1H NMR spectra for amide **5** at different pH^* values. Smaller changes are observed for the chemi-

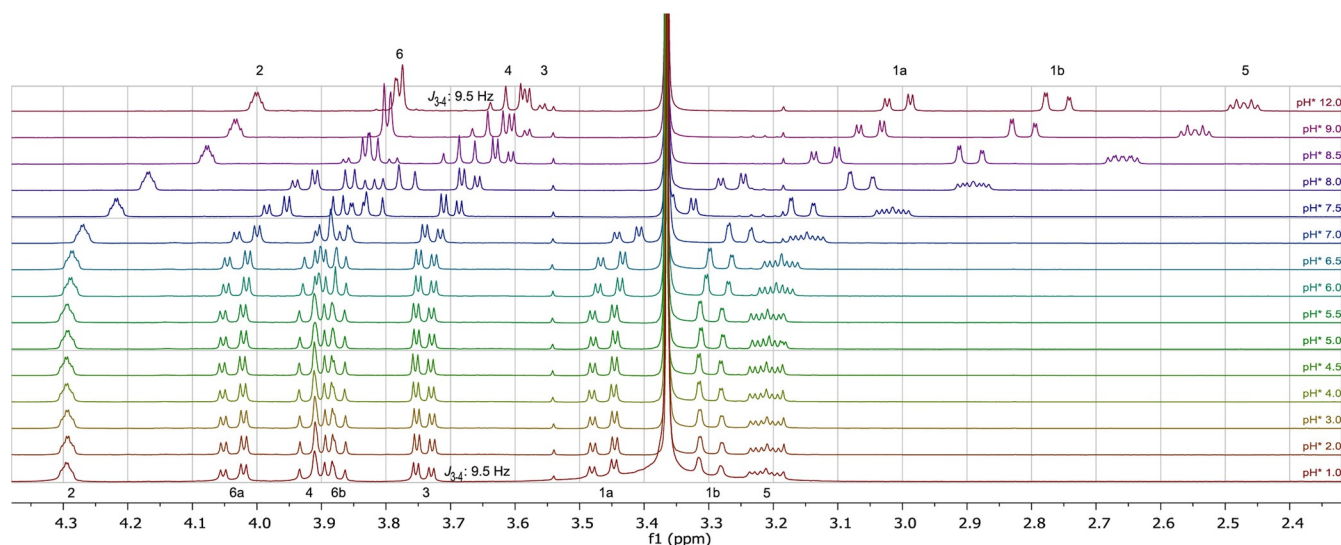


Figure 1. 400 MHz 1H NMR spectra for DMJ (**2**) at different pH^* values; spectra are referenced to residual methanol.

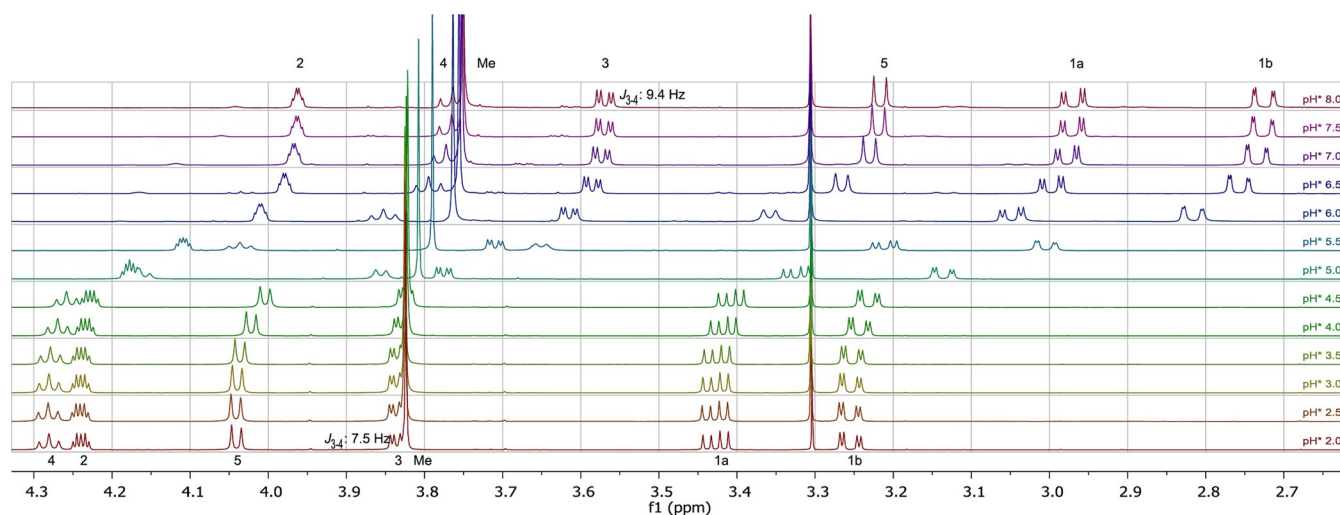


Figure 2. ^1H NMR spectra for 2,6-dideoxy-2,6-iminomannuronic acid methyl ester (3) at different pH^* values; spectra are referenced to residual methanol.

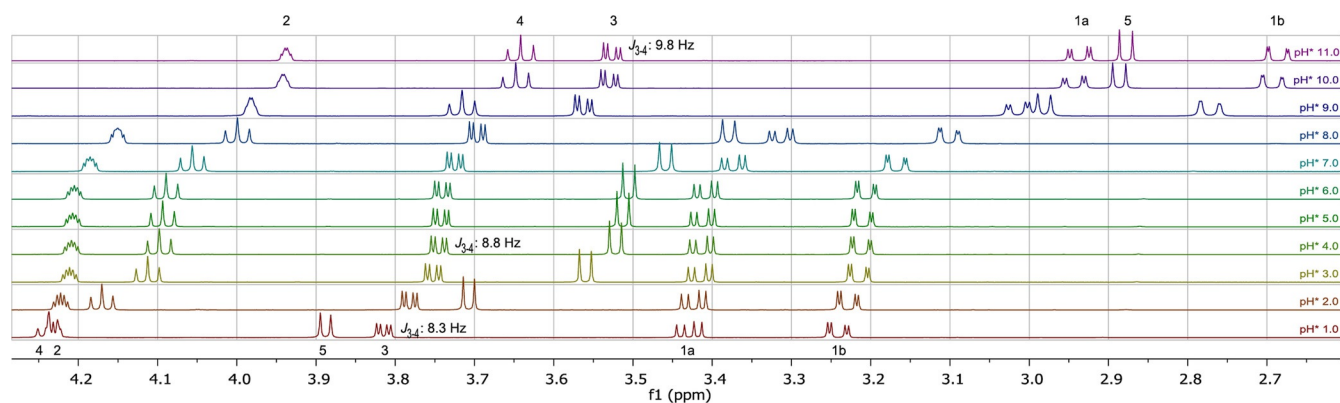


Figure 3. ^1H NMR spectra for 2,6-dideoxy-2,6-iminomannonic acid (4) at different pH^* values; spectra are referenced to water.

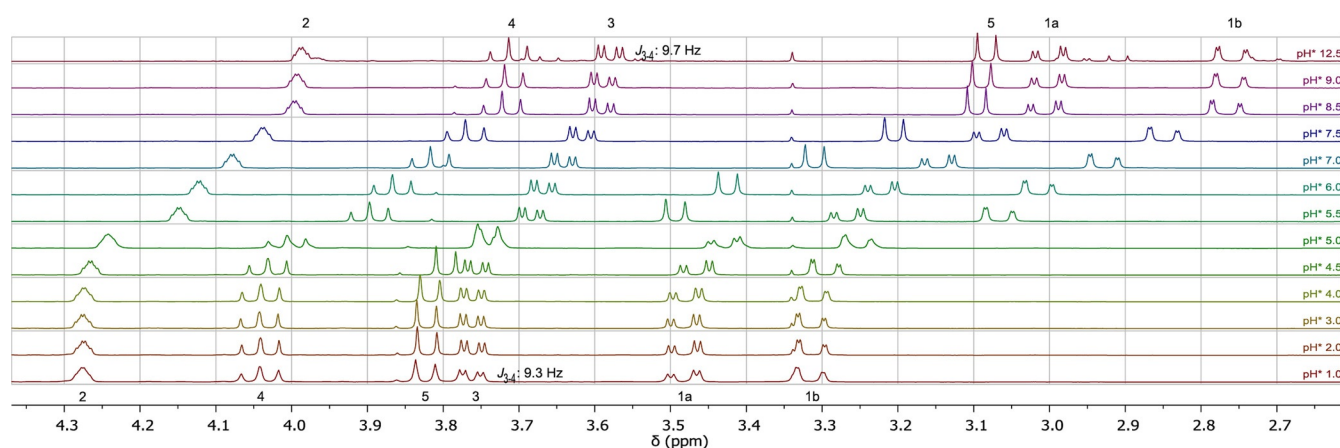


Figure 4. ^1H NMR spectra for 2,6-dideoxy-2,6-iminomannuronic amide (5) at different pH^* values; spectra are referenced to water.

cal shift change of H5 and there is no significant change in the coupling constants, thus indicating minimal conformation changes on going from high to low pH^* for this azasugar.

To establish the ratio of $^1\text{C}_4$ and $^4\text{C}_1$ conformers for the different azasugars we used DFT calculations to determine the coupling constants of the two conformers of both the protonated

and the deprotonated azasugars (for details see the Supporting Information).^[17] Table 1 shows the measured coupling constants ($J_{3,4}$) for the four azasugars at low and high pH^* values, the calculated $J_{3,4}$ values for the $^4\text{C}_1$ and $^1\text{C}_4$ conformers and the ratios of the two conformers, established from the measured average coupling constants. As can be seen from

Table 1, there is good agreement between the calculated and the measured coupling constants at high pH* values. With the two values for $J_{3,4}$ the ratios of the 1C_4 and 4C_1 conformers were established. It is clear that DMJ (2) exists in a single 4C_1 conformation at both low and high pH values. For the methyl ester 3 the situation is different. With the calculated values for the coupling constants of both conformers ($J_{3,4}$ = 9.5 Hz and 4.9 Hz for the 1C_4 and 4C_1 azasugars, respectively) and the measured average coupling constant ($J_{3,4}$ = 7.5 Hz) the ratio of the two conformers was established to be 56:44, thus indicating that the two chair conformers are equally stable. In similar vein, the ratio of the two chair conformers of the acid 4 was determined at three different pH values. As can be seen in Table 1, at high pH the anionic azasugar 4 exists as a single conformer, whereas at pH 5 the measured average coupling constant indicates a 94:6 mixture of conformers. At low pH the two conformers are observed in a 75:25 4C_1 / 1C_4 ratio. For the amide 5, at both high and low pH the 4C_1 chair is almost exclusively present.

To investigate the conformational behaviour in a less polar environment, the azasugar showing the largest conformational change, methyl ester 3, was investigated in CD_3OD . Figure 5 shows the spectra of the non-protonated and the protonated azasugar. In this medium the $J_{3,4}$ coupling constant changes from 9.2 Hz to 4.8 Hz upon protonation, thus indicating that the non-protonated azasugar exists in the 4C_1 conformation whereas the protonated species is found in the opposite 1C_4 conformation.

The NMR results show that DMJ analogues possessing a methyl ester or carboxylic acid at C5 (as in 3 and 4, respectively) can change their conformation from the 4C_1 chair form to the opposite 1C_4 chair upon protonation. This conformational change is seen even in a highly polar medium such as water and is significantly enhanced in a more apolar solvent (CD_3OD). The nature of the substituent at C5 of the DMJ analogues is of major importance, because DMJ (2) and its C5 amide 5 do not display any conformational change with changing pH. The difference between the ester and amide is notable, because both functional groups—the C5 carboxylic acid ester and the C5 carboxamide—have a similar effect on

the basicity of the azasugars. The electron-withdrawing effect of both groups leads to a significant drop in the pK_a values for 3 and 5, with the more strongly electron-withdrawing functionality—the ester—having the stronger inductive effect. The conformational flip of ester 3 and acid 4 can be accounted for by considering that electron-withdrawing groups prefer to occupy an axial position on a positively charged pyranose ring to minimise their destabilising effect.^[4–7] The fact that amide 5 does not change its conformation to accommodate this intrinsic preference might be due to internal hydrogen bonds that can be formed between the amide $-NH_2$ and the C4-OH, which provides an extra stabilising factor in the 4C_1 amide.^[18]

Having established that the mannuronic acid azasugars readily undergo ring flip upon protonation we probed the binding of the azasugars in the binding pocket of the α -1,2-mannosidase GH47, from the *Caulobacter* K31 strain. All four compounds were tested for binding through X-ray crystallography and isothermal titration calorimetry. Initially we analysed the binding of the parent compound DMJ (2).

DMJ (2) binds to CkGH47 with a K_D of 481 nM (determined by isothermal titration calorimetry, Figure 6A). Although DMJ binding is essentially as observed previously for the mammalian GH47 structures,^[19] the subatomic resolution data (Supporting Information) in this case allow us to observe DMJ bound in the active site of CkGH47 in two different ring conformations (Figure 6B). In the -1 subsite, the conformation of DMJ is in both 3S_1 and 1C_4 , each with a modelled occupancy of 0.5. Both conformations are consistent with the conformational itinerary of GH47 in which the structure adopts a 3S_1 conformation in the Michaelis complex to react via a 3H_4 transition state to form the product in a 1C_4 conformation.^[12, 19, 20] This dual-conformation observation could perhaps be explained by the proximity of the pH of the crystallisation conditions (6.5) to the pK_a of DMJ (7.5) and the protonation of the species, although one cannot deconvolute which conformer relates to which protonation state).

The structure (PDB ID: 5MEH) confirms proposals made by others, and by us, concerning the catalytic apparatus.^[12, 19, 20] Briefly, catalytic base E365 is hydrogen-bonded to the O6 of

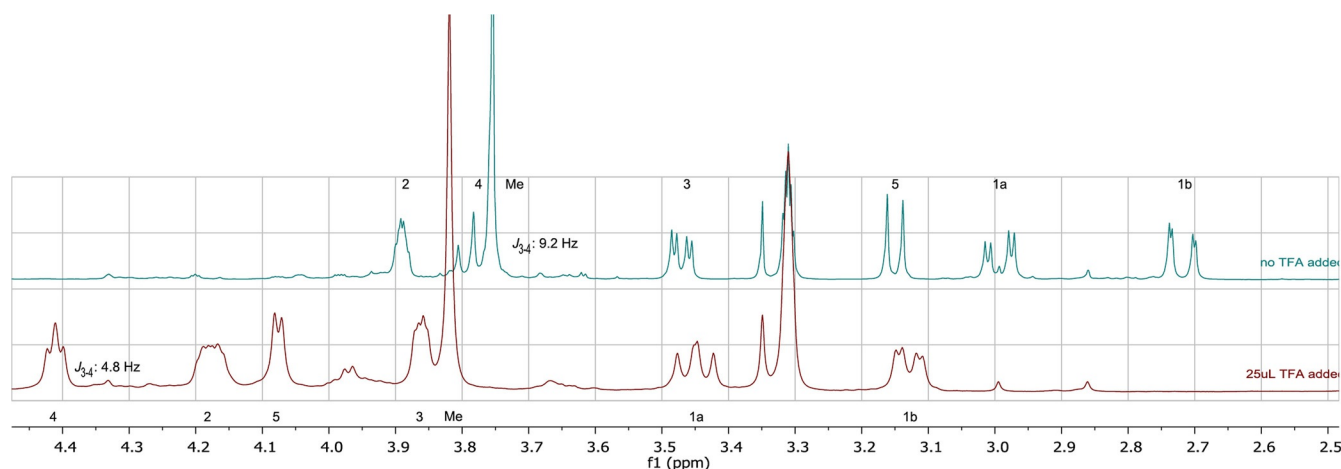


Figure 5. DMJ methyl ester (3) in CD_3OD (≈ 0.7 mL). The non-protonated azasugar is shown on top (no TFA added), the protonated azasugar on the bottom (25 μ L TFA added). Spectra are referenced to CD_2HOD .

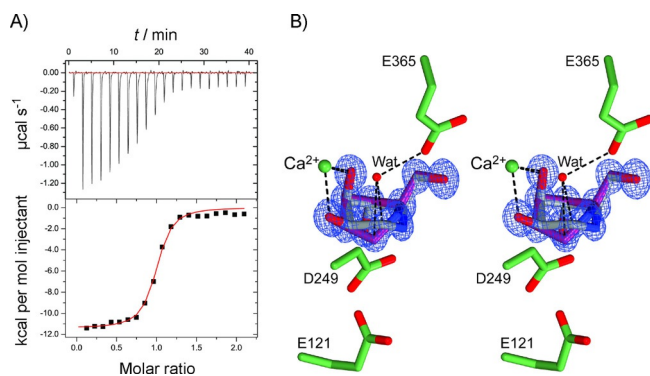


Figure 6. Binding of DMJ (**2**) to CkGH47. A) ITC-derived thermodynamics of binding. The stoichiometry (n) was 0.96 ± 0.01 sites. The association constant (K_A) was $(2.1 \times 10^6 \pm 3.3 \times 10^5) \text{ M}^{-1}$. The enthalpy change (ΔH) was $(1140 \pm 150) \text{ cal mol}^{-1}$. B) Divergent wall-eyed stereo electron density for the structure of CkGH47 in complex with DMJ (**2**, with the two conformations shown with grey/purple bonds). The map shown is a maximum-likelihood/ σ_A weighted $2F_{\text{obs}} - F_{\text{calc}}$, contoured at $0.87 \text{ electrons } \text{\AA}^{-3}$. The active centre calcium is shown as a green sphere and a water (Wat)—likely equating to the nucleophilic water in catalysis—is shown as a red sphere. Key active centre residues discussed in the text are labelled.

DMJ, at 2.6 \AA . It is held in place by the nucleophilic water, which in turn is coordinated by calcium. The indirect route to protonic assistance is likely given by the O ϵ 2 of E121, although a role for D249 has also been considered. The riding hydrogens of this bond are visible, matching the level of detail of structure achieved by Thompson et al.^[12a] With the assistance of a metal ion, the O2-C2-C3-O3 torsion angle of a ${}^4\text{C}_1$ conformation is tightened from $\approx 60^\circ$ to $0\text{--}15^\circ$, consistent with the known conformational pathway of GH47 via a ${}^3\text{H}_4$ transition state.^[12, 19, 20]

Unfortunately, despite promising solution characteristics (Table 1), we were not able to detect binding of mannuronic acid derivatives **3**, **4** or **5**. Simple modelling of these compounds in the active centre, using the DMJ complex as a template, suggested that the likely reason would be steric clashes with residues in the active site, in particular E427. To test this hypothesis, a mutant containing an E427A mutation to increase the size of the active site was produced; however, attempts to obtain complexes with this variant still did not allow observation of **3–5** in the -1 subsite of the enzyme (data not shown), thus suggesting that further steric clashes might also be contributing to the lack of inhibition.

Conclusion

Azasugars based on mannuronic acid can change their conformation from a “normal” ${}^4\text{C}_1$ chair to the inverted ${}^1\text{C}_4$ chair form upon protonation of the endocyclic amine. The molecules thereby position their substituents such that they are optimally positioned to accommodate the positive charge. Although the conformational behaviour of other glycuronic-acid-based azasugars with different substituent configurations has not yet been studied in detail, it is likely that the spatial preferences of the substituents in the mannuronic acid azasugar work in concert to affect the ring flip. This behaviour is in line with the

conformational effects observed for fully protected mannuronic acid glycosyl donors, and therefore the results described here provide an extra indication that the positive charge at the anomeric centre of a mannuronic acid oxocarbenium ion is responsible for the observed unusual ring flip. This intriguing ring-flipping behaviour pointed to the potential use of the mannuronic acid azasugars as inhibitors for mannosidases that hydrolyse their substrates through a ring-flipped conformational itinerary. Unfortunately, the mannuronic acid azasugars did not bind to the studied GH47 mannosidase. Although the concept of chemically flipped inhibitors worked in solution, especially in low-polarity buffers, they sadly highlight the challenges of conformationally specific enzyme inhibition. For, whilst the introduction of favourable chemistry—including, in some cases, locking groups—frequently introduces substituents that prevent binding for steric reasons, in enzyme active centres that have evolved to harness the interactions of and thus distort unsubstituted sugars (e.g., the elegant locking of a mannoside mimic into $B_{2,5}$ conformation with a three-carbon bridge^[21]—in order to target $B_{2,5}$ transition-state mannosidases specifically) it simply resulted in steric clashes with the target β -mannosidase and no inhibition of the wild-type enzyme.^[22] Indeed, although the concept of conformation-specific targeted inhibition is one of the most exciting in glycochemistry, it is only rarely achieved: the use of ring-flipped kifunensine (**1**) to inhibit “southern hemisphere” mannosidases is one of the very few cases in which a conformationally restrained inhibitor works (and has indeed found considerable application in cell biology).^[23] The challenge therefore is still to provide the specific tools and therapeutic compounds required for cellular or patient use, whilst also maintaining binding to the target enzyme.

Experimental Section

General methods for organic synthesis: All reagents were of commercial grade and used as received unless stated otherwise. Reactions were performed at room temperature unless stated otherwise. Molecular sieves (4 \AA) were flame-dried before use. Flash column chromatography was performed on silica gel ($40\text{--}63 \text{ }\mu\text{m}$). ${}^1\text{H}$ and ${}^{13}\text{C}$ NMR spectra were recorded with Bruker AV 600, Bruker AV 400 or Bruker DPX 400 spectrometers in D_2O or CD_3OD . Chemical shifts (δ) are given in ppm relative to the solvent residual signals. Coupling constants (J) are given in Hz. All given ${}^{13}\text{C}$ spectra are proton-decoupled. Compound names are given with use of the standard iminosugar nomenclature numbering.

2,6-Dibromo-2,6-dideoxy-D-mannono-1,4-lactone (7**):** Calcium D-gluconate monohydrate (**6**, 126 g , 280 mmol) was put under argon before being dissolved in 33% HBr in acetic acid (500 mL , 3.0 mol). The reaction mixture was stirred for 18 h to give an acetylated form of **6**. MeOH (1 L) was added, and the mixture was heated at reflux for 2 h. It was then concentrated to half its original volume under reduced pressure before addition of more MeOH (500 mL). The reaction mixture was left to stir overnight, after which it was concentrated, resulting in a slightly oily residue. This was co-evaporated with MeOH (100 mL) and three times with H_2O (100 mL). The residue was extracted with diethyl ether ($4 \times 100 \text{ mL}$), and the organic layers were combined, dried with MgSO_4 , filtered and concentrated under vacuum, yielding a yellow oily residue. This was

crystallised from $\text{CHCl}_3/\text{H}_2\text{O}$ to yield a white crystalline solid (44 g, 146 mmol, 26% yield). M.p. 130°C ; $[\alpha]_{\text{D}}^{20} = +58.6^\circ$ ($c=1$, MeOH); ^1H NMR (400 MHz, D_2O): $\delta=5.20$ (d, $J=4.5$ Hz, 1H; C-2), 4.64 (m, 2H; C-4, C-3), 4.19 (m, 1H; C-5), 3.77 (dd, $J=11.4$, 2.4 Hz, 1H; C-6a), 3.65 ppm (dd, $J=11.4$, 4.9 Hz, 1H; C-6b); ^{13}C NMR (101 MHz, D_2O): $\delta=174.0$ (C-1), 81.6 (C-4), 69.1 (C-3), 66.2 (C-5), 47.6 (C-2), 36.6 ppm (C-6).

2-Amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (8): 2,6-Dibromo-2,6-dideoxy-D-mannono-1,4-lactone (7, 5.0 g, 16.5 mmol) was put under argon and dissolved in dry acetone (MgSO_4 , 100 mL). Sodium azide **Caution: highly toxic** (15.0 g, 231 mmol) was added, and the suspension was heated at reflux for 20 h. The mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was dissolved in H_2O (50 mL) and extracted with diethyl ether (5×100 mL), and the organic layers were combined, dried over MgSO_4 , filtered and concentrated under reduced pressure to give a brown oil that was identified as the 2-azido compound but included some of its diastereoisomer. ^1H NMR (400 MHz, D_2O): $\delta=4.68$ (dd, $J=4.5$, 3.3 Hz, 1H; C-3), 4.56 (d, $J=4.6$ Hz, 1H; C-2), 4.46 (dd, $J=9.2$, 2.7 Hz, 1H; C-4), 4.09 (m, 1H; C-5), 3.69 (dd, $J=11.4$, 2.7 Hz, 1H; C-6a), 3.56 ppm (dd, $J=11.5$, 4.9 Hz, 1H; C-6b); ^{13}C NMR (101 MHz, D_2O): $\delta=174.1$ (C-1), 81.0 (C-4), 69.6, 65.7 (C-3, C-5), 62.3 (C-2), 36.6 ppm (C-6).

The crude compound (16.5 mmol) was put under argon and dissolved in MeOH (100 mL). Palladium on activated carbon (10%, 300 mg, 0.3 mmol) and HCl (37% in H_2O , 10 mL, 121 mmol) were added, and the suspension was put under hydrogen. The reaction mixture was stirred for 22 h, after which the catalyst was filtered off over a Whatman microfilter. The filtrate was concentrated under reduced pressure and co-evaporated once with HCl (37% in H_2O , 60 mL), thrice with toluene (60 mL) and once with CHCl_3 (50 mL). Crystallisation from EtOH yielded 2-amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (8) as white crystals (2.6 g, 9.2 mmol, 55% over two steps). M.p. 207°C (decomp.); $[\alpha]_{\text{D}}^{20} = +41.6^\circ$ ($c=1$, MeOH); ^1H NMR (400 MHz, D_2O): $\delta=4.83$ (dd, $J=4.8$, 2.8 Hz, 1H; C-3), 4.63 (dd, $J=9.2$, 2.7 Hz, 1H; C-4), 4.59 (d, $J=4.9$ Hz, 1H; C-2), 4.20 (m, 1H; C-5), 3.77 (dd, $J=11.5$, 2.6 Hz, 1H; C-6a), 3.65 ppm (dd, $J=11.4$, 5.0 Hz, 1H; C-6b); ^{13}C NMR (101 MHz, D_2O): $\delta=172.1$ (C-1), 78.9 (C-4), 64.1 (C-3), 62.9 (C-5), 50.2 (C-2), 33.7 ppm (C-6).

1-Deoxymannojirimycin (2): 2-Amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (8, 501 mg, 1.8 mmol) was co-evaporated three times with dry toluene, put under argon and suspended in dry MeOH (10 mL). The suspension was cooled to 0°C before addition of distilled triethylamine (1.0 mL, 7.2 mmol), and the resulting clear solution was stirred overnight. The reaction mixture was concentrated under reduced pressure, yielding the methyl ester as a white semicrystalline solid. The residue was put under argon, dissolved in dry EtOH (molsieves, 10 mL) and cooled to 0°C . Sodium borohydride (709 mg, 19 mmol) was added, and the suspension was stirred overnight. Dry MeOH (20 mL) was added, after which the mixture was filtered, concentrated under reduced pressure and co-evaporated with HCl in MeOH (1 M, 3×10 mL). The residue was purified by column chromatography (EtOAc/EtOH 1:1 \rightarrow 100% EtOH), yielding a pure sample of DMJ (2) in 29% yield (105 mg, 0.50 mmol). $[\alpha]_{\text{D}}^{20} = -14.0^\circ$ ($c=0.5$, MeOH); ^1H NMR (399 MHz, D_2O): $\delta=3.99$ (dt, $J=2.9$, 1.6 Hz, 1H; C-3), 3.76 (dd, $J=12.5$, 3.9 Hz, 1H; C-7), 3.71 (dd, $J=12.5$, 5.5 Hz, 1H; C-7a), 3.60 (t, $J=9.7$ Hz, 1H; C-5), 3.53 (dd, $J=9.6$, 3.1 Hz, 1H; C-4), 3.03 (dd, $J=14.2$, 2.8 Hz, 1H; C-2a), 2.80 (dd, $J=14.2$, 1.5 Hz, 1H; C-2b), 2.57 ppm (ddd, $J=9.7$, 4.9, 3.4 Hz, 1H; C-6); ^{13}C NMR (101 MHz,

D_2O): $\delta=74.4$ (C-4), 67.6 (C-5), 67.5 (C-3), 62.4 (C-6), 59.6 (C-7), 48.9 ppm (C-2).

Methyl 2,6-dideoxy-2,6-imino-D-mannonate hydrochloride (3): 2-Amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (8, 0.60 g, 2.2 mmol), was co-evaporated thrice with dry toluene, put under argon and suspended in dry MeOH (12 mL). The suspension was cooled to 0°C before addition of distilled triethylamine (1.2 mL, 8.7 mmol), and the resulting clear solution was stirred overnight. The reaction mixture was concentrated under reduced pressure before being taken up in acetonitrile (15 mL) and charged with 1,1,1,3,3,3-hexamethyldisilazane (2.5 mL, 12 mmol) and copper sulfate pentahydrate (cat.). After 1 h, the reaction mixture was concentrated and a fraction of 234 mg (0.57 mmol) was purified by column chromatography (1–2.5% 1,4-dioxane/ CH_2Cl_2) to give the per-TMSylated compound (162 mg, 0.40 mmol). The protected product was put under argon and dissolved in MeOH (8 mL), and acetyl chloride (1 equiv) was added to generate HCl in situ. The mixture was stirred for 0.5 h, after which the compound was concentrated and co-evaporated with MeOH to yield the title compound (98 mg, 0.40 mmol, 70% over two steps). $[\alpha]_{\text{D}}^{20} = +31.8$ ($c=1$, MeOH); ^1H NMR (400 MHz, D_2O): $\delta=4.40$ (dd, $J=5.3$, 4.8 Hz, 1H; C-5), 4.17 (ddd, $J=9.5$, 4.1, 2.8 Hz, 1H; C-3), 4.09 (d, $J=4.4$ Hz, 1H; C-6), 3.86 (dd, $J=5.6$, 2.7 Hz, 1H; C-4), 3.82 (s, 3H; OCH_3), 3.44 (dd, $J=12.2$, 9.6 Hz, 1H; C-2a), 3.13 ppm (dd, $J=12.2$, 4.2 Hz, 1H; C-2b); ^{13}C NMR (101 MHz, D_2O): $\delta=168.1$ (C-7), 71.1 (C-4), 69.9 (C-5), 64.2 (C-3), 58.8 (C-6), 53.4 (OCH_3), 43.5 ppm (C-2).

Sodium 2,6-dideoxy-2,6-imino-D-mannonate (4): Methyl 2,6-dideoxy-2,6-imino-D-mannonate hydrochloride (3, 24 mg, 0.10 mmol) was dissolved in H_2O (0.5 mL). A sodium hydroxide solution (1 M aq., 170 μL , 0.17 mmol) was added, and the mixture was stirred for 2 h. The mixture was concentrated under reduced pressure to yield the title compound, pure but with added sodium hydroxide. $[\alpha]_{\text{D}}^{20} = -7.2$ ($c=1$, MeOH); ^1H NMR (400 MHz, D_2O): $\delta=4.01$ (m, 1H; C-3), 3.71 (t, $J=9.7$ Hz, 1H; C-5), 3.60 (dd, $J=9.6$, 3.2 Hz, 1H; C-4), 3.01 (dd, $J=14.6$, 2.7 Hz, 1H; C-2a), 2.95 (d, $J=9.8$ Hz, 1H; C-6), 2.75 ppm (dd, $J=14.6$, 1.6 Hz, 1H; C-2b); ^{13}C NMR (101 MHz, D_2O): $\delta=178.4$ (C-7), 74.1 (C-4), 70.6 (C-5), 69.1 (C-3), 65.2 (C-6), 47.9 ppm (C-2).

2,6-Dideoxy-2,6-imino-D-mannonic amide (5): 2-Amino-6-bromo-2,6-dideoxy-D-mannono-1,4-lactone hydrochloride (8, 500 mg, 1.8 mmol), was co-evaporated thrice with dry toluene, put under argon and suspended in dry MeOH (10 mL). The suspension was cooled to 0°C before addition of distilled triethylamine (1.0 mL, 7.2 mmol), and the resulting clear solution was stirred overnight. The reaction mixture was concentrated under reduced pressure, yielding the methyl ester as a white semicrystalline solid. The residue was dissolved in ammonia in MeOH (6 M, 10 mL, 60 mmol) and stirred overnight. The reaction mixture was concentrated under reduced pressure, affording 2,6-dideoxy-2,6-imino-D-mannonic amide (5) in quantitative yield. An analytical sample was prepared by crystallisation from pure MeOH (133 mg, 0.76 mmol, 42%). $[\alpha]_{\text{D}}^{20} = -31.6^\circ$ ($c=0.5$, H_2O); ^1H NMR (399 MHz, D_2O): $\delta=3.97$ (m, 1H; C-3), 3.70 (t, $J=9.7$ Hz, 1H; C-5), 3.57 (dd, $J=9.6$, 3.1 Hz, 1H; C-4), 3.07 (d, $J=9.8$ Hz, 1H; C-6), 2.99 (dd, $J=14.6$, 2.7 Hz, 1H; C-2a), 2.75 ppm (dd, $J=14.6$, 1.6 Hz, 1H; C-2b); ^{13}C NMR (101 MHz, DMSO): $\delta=173.5$ (C-7), 74.9 (C-4), 70.0 (C-5), 68.7 (C-3), 63.3 (C-6), 49.1 ppm (C-2); HRMS: m/z calcd for $\text{C}_6\text{H}_{12}\text{O}_4\text{N}_2$: 177.08698 $[\text{M}+\text{H}]^+$; found: 177.08683.

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Conflict of Interest

The authors declare no conflict of interest.

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